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Quantitative Imaging of Single mRNA Splice Variants in Living Cells

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Abstract

Alternative mRNA splicing is a fundamental process of gene regulation via the precise control of the post-transcriptional step that occurs before mRNA translation. Errors in RNA splicing have been known to correlate with different diseases; however, a key limitation is the lack of technologies for live cell monitoring and quantification to understand the process of alternative splicing. Here, we report a spectroscopic strategy for quantitative imaging of mRNA splice variants in living cells, using nanoplasmonic dimer antennas. The spatial and temporal distribution of three selected splice variants of the breast cancer susceptibility gene, BRCA1 were monitored at single copy resolution by measuring the hybridization dynamics of nanoplasmonic antennas targeting complementary mRNA sequences in live cells. Our study provides valuable insights on RNA and its transport in living cells, which has the potential to enhance our understanding of cellular protein complex, pharmacogenomics, genetic diagnosis, and gene therapies.

Keywords

mRNA detection; splicing; single molecule detection; hyperspectral intracellular imaging; transcript quantification; single cell/molecule imaging

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AUTHOR CONTRIBUTIONS

K.L. and J.I. conceived the original idea for this study. K.L., L.L., and J.I. designed the experiments. K.L. built the hyperspectral imaging system and developed the data analysis algorithm. K.L. performed the nanoparticle dimer probe design/synthesis/characterization, microinjection, and optical measurements. Y.C. performed the PCR, flow cytometry and other supporting biological validations. K.L., L.L., and J.I. analyzed the data and wrote the manuscript.

SUPPLEMENTARY INFORMATION AVAILABLE

Details on the spectral description of nanoparticle structures, custom built hyperspectral imaging system, signal analysis protocol, *in vitro/vivo* control and validation experiments are presented. Movie files of single molecule hybridization and time-resolved intracellular mRNA splicing variant tracking are also presented. Supplementary information accompanies this paper at www.nature.com/naturenanotechnology. Reprints and permission information is available online at <http://npg.nature.com/reprintsandpermissions/>. Correspondence and requests for materials should be addressed to J.I. and L.L.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Alternative transcripts enable generation of multiple mRNA isoforms from single genes; more than 90% of human genes undergo alternative splicing. Although their expression level is generally low, mRNA splice variants are critical for cell growth and development; defects in the expression of specific isoforms can lead to abnormal gene expression, cellular dysfunction, and disease. Breast cancer susceptibility gene 1 (BRCA1) plays a significant role in formation of sporadic tumors in the absence of germline mutations^{1,2} and is used as an indicator of malignant breast cancer. However, the regulation, localization, and relative abundance of BRCA1 splicing transcripts in different cell types, as well as the contribution of the translated protein isoforms to cell growth, are poorly characterized. Northern blots³ and reverse transcription polymerase chain reaction (RT-PCR)⁴ are commonly used to detect specific mRNA transcripts; however, these methods require millions of cells and are not applicable to real-time studies of live single cells. Fluorescence^{5–8}, molecular beacons⁹, Förster resonance energy transfer (FRET)^{10,11}, and fluorescently labeled modified metal nanoparticles^{12–14} have also been used to detect mRNA in living cells; however, these methods lack the flexibility to detect small gene sequence variations¹⁵. Therefore, detection and quantification of mRNA splicing variants in living cells are currently challenging.

Gold nanoparticle (GNP) conjugated DNA probes hybridized to target molecules are of significant interest owing to their strong plasmonic signals and probe stability, which enable intracellular single particle detection^{16,17} without notable degradation.¹⁴ During interaction with photons, the plasmonic cross section generates a 10- to 10⁴-fold stronger signal than fluorophores or quantum dots, while still retaining well-defined optical characteristics.^{18–22} Relative to individual GNPs, network structures formed by target molecule binding display enhanced surface-enhanced Raman scattering signal (SERS)²³ and Rayleigh scattering²⁴, making them ideal candidates for single molecule probes. To this end, the plasmon resonance of noble metal nanoparticles in the visible range has been utilized to detect particle structures^{20,25–27} or nanoparticle dimer formation.^{28,29} In particular, when a dimer with less than 3 nm of inter-particle spacing is formed as a result of two probes binding to a single target molecule, the signal intensity of the single dimer is strong enough to allow intracellular imaging due to local field enhancement. Hence, nanoplasmonic dimer-based approaches using dark-field microscopy are powerful tools for detecting molecules such as DNA³⁰ and protein^{31,32}. However, achieving the sensitivity required to detect and quantify single molecules in living cells is challenging because of the inability to discriminate specific signals from the background noise due to the intracellular environment.

Here, we present an elegant plasmonic nanoparticle network structure-based approach, which generates a plasmon-coupled dimer capable of detecting single mRNA variants. This method was used to detect and quantify BRCA1 mRNA splice variants both *in vitro* and *in vivo*. Two nanoparticle-conjugated probes were attached to the BRCA1 mRNA target in a sequence-specific manner, resulting in a spectral shift of the signal due to dimer formation. This method is powerful and as demonstrated can successfully detect, quantify, and differentiate between multiple BRCA1 splice variants with single copy sensitivity, paving the way for single cell quantitative genetic profiling in the long-term.

Nanoplasmonic dimers for detection of mRNA splice variants

Experiments described in our study were performed using DNA probes targeting specific sequences within the BRCA1 mRNA, conjugated to 40 nm GNPs. Hybridization of these probes to a single target mRNA resulted in the formation of nanoparticle dimers with small inter-particle distances producing spectral peak shift due to strong plasmonic coupling (Figure 1a). As shown in Figure 1a i) and ii), dimer formation caused spectral color and intensity changes indicating the identification of one copy of the target mRNA. The location and number of dimers, as determined by the signal intensity and spectral peak shift, could be used to track and quantify single mRNAs using dark-field hyperspectral images. The signal intensities of the dimers were many folds greater than those generated by monomer probes. In addition, the nanoparticle interactions could occur over much longer distances than those achieved by other probes due to the rich conductive electrons, such as fluorescent moieties or quantum dots. In essence, the dimer strategy enabled detection of mRNA splice variants via analysis of spectral peak shifts that depend on inter-particle distances between probes (see Supplementary Information SI1). The proposed strategy is therefore suitable for detection of alternative splicing variants, where hundreds of base pairs of sequence might be spliced out (Figure 1b). Distinct spectral peak shifts and intensity changes were observed over inter-particle distances up to approximately 30 nm when nanoparticle dimers were used, posing an exclusive advantage of plasmonic dimer over other conventional methods. Our observation of the spectral peak shifts and intensity changes agrees well with others' reports^{33,34}. Figure 1b shows the difference in the interparticle distances of the nanoparticle dimers attached to mRNA wild-type and its splice variants due to the missing sequences, resulting in spectral peak shifts in their signals. Consequently, the nanoparticle dimer formation approach has tremendous advantages for live cell monitoring of mRNA variants and quantification in the time-resolved images.

In vitro quantification of mRNA hybridization

Based on the single particle sensitivity of GNP-conjugated sensors previously reported *in vitro*²⁹ and *in vivo*¹⁷, hybridization dynamics of nanoparticle-conjugated sensors *in vitro* were characterized by colorimetric imaging and spectral quantification, using probes specific to the sequence in BRCA1 mRNA (Figure 2). To monitor hybridization dynamics and to quantify the number of dimers formed, colorimetric images were obtained at a temporal resolution of 1 frame per second. The number of dimers in the colorimetric images (Figure 2a) was compared with the results of spectral quantification (Figures 2b and 2c), which is directly proportional to the number of nanoparticles, according to the Mie theory³⁵ and static light scattering.³⁶ Following introduction of the oligonucleotide sequence representing a segment of BRCA1 mRNA splice junction, the monomer particles (Figure 2a; not clear in the image due to the weak intensity and the fast dynamics) formed dimers (Figure 2a; indicated by high intensity dots) in a time-dependent manner. The background in each of the images became brighter across time because the scattering intensity of the nanoparticle dimer was approximately 4.7-fold higher than that of the monomer. In the absence of the target sequence, no changes in colorimetric images were detected for up to 2 hours. Further, no changes were observed when non-specific sequences were used as negative control (Supplementary Information SI6). In the region of the image shown in

Figure 2a (red box), seven nanoparticle dimers appeared during the 24 min measurement window, while monomer intensity equivalent to 16 monomers decreased, yielding a ratio of approximately 2.3 monomers per dimer. These experiments demonstrated that a dimer was formed by the assembly of two monomers onto a single target mRNA *in vitro*, and illustrated the suitability of this technique for imaging and quantification of mRNA. Further demonstration of multiplex detection is described in the Supplementary Information (SI7).

As mentioned above, the spectra in a sample volume fraction of 340 fL (determined by the measurements in 4×1 pixels) were also measured to statistically quantify the formation of dimers. The monomer and dimer peak intensities at 538 nm (Figure 2b) and 612 nm (Figure 2c), respectively, displayed trends similar to those observed by colorimetric imaging. Specifically, the intensity distribution at 538 nm decreased over time whereas the intensity distribution at 612 nm increased over time. The spectral data for the monomer peak indicated a decrease of approximately 64 particles, as approximately 37 dimers were formed in the sample. These data confirm that dimers were formed from monomers as a result of hybridization to the target transcripts (Figure 2d). Distributions of monomer and dimer intensity broadened over time and were binomial. The result was in agreement with the data obtained from the colorimetric imaging method, demonstrating that quantification and imaging are complementary techniques.

To confirm detection of dimers at the single molecule level, we incubated 30 fM of the oligonucleotide sequence representing a segment of BRCA1 mRNA splice junction with 5'-nanoparticle monomers and immobilized complementary 3'-nanoparticle monomers on a glass surface. Single dimer formation was observed by colorimetric imaging (Figure 2e), verifying that a single mRNA molecule could be detected (see Movie in the Supplementary Information). The hybridization mechanism was also confirmed by a melting temperature assay. At the melting temperature of the target sequence (64.7°C), the spectral peak of dimers (612 nm) reverted back to the monomer peak position (538 nm), verifying that the nanoparticle dimer was formed by hybridization (Figure 2f). Similar phenomenon was observed in the nanoparticle dimer dehybridization experiment (Supplementary Information SI11) confirming the sensitivity of the approach.

Hyperspectral imaging of intracellular single mRNA *in vivo*

We then investigated the potential of plasmonic dimer method for imaging and quantification of intracellular single mRNAs *in vivo*. While the *in vitro* nanoparticle signals could easily be normalized to the uniform halogen lamp background, the *in vivo* signals have to be normalized against the heterogeneous signal from the cellular background. The background signal of *in vivo* measurements is dependent on the particular cell line, cellular growth stage, and physiological conditions; therefore, the major differences between *in vitro* and *in vivo* measurements are in the complexity of instrumentation to exclude the strong spectral background and analysis algorithm to maximize the signal-to-noise ratio (SNR) (Supplementary Information SI4). For this reason, the hyperspectral imaging strategy with optimized analysis algorithm was used to capture the full spectrum at each spot in the image and to differentiate between the presence or absence of monomers and dimers inside the cell with detectable SNR.

Quantification of intracellular dimers was demonstrated by injecting known concentrations of dimers pre-assembled by the hybridization of probe sequences (PS) and target sequence (TS) (assembly of GNP-PS1, GNP-PS2, and TS1) into live cells (Figure 3a). Microinjection was chosen as the probe delivery method because it allows fine control of the various parameters, such as injection volume and injection time, for direct observation of the injected cells.³⁷ By virtue of injection volume control, appropriate calibration standards could be developed for the first time for the intracellular quantification of transcripts in live cells (Figure 3). Figure 3a i)-iv) shows that approximately the same number of injected dimers were detected in the dimer localization map from hyperspectral imaging, where about 12.5, 25, 37.5, or 50 dimers were introduced into different cells. After injection, normal dark-field and hyperspectral images corresponding to the 612 nm wavelength were reconstructed; as expected, the images clearly showed a concentration-dependent increase in the number of dimers inside the cells (Figure 3a). The same result was obtained using the ensemble method of spectral quantification of monomers and dimers in the mixture injected into individual cells (Figure 3b). Control experiments comprised of cells that did not contain nanoparticles or that were injected with nanoparticle monomers without targets (GNP-PS1 and GNP-PS2 without TS1), which did not show any detectable dimer signal either from imaging or in the spectral quantification calculations (Figure 3b inset, Supplementary information SI8). These experiments show that the formation of nanoparticle dimers in live cells can be monitored and that the quantity and localization of these dimers can be pinpointed.

BRCA1 gene encodes a nuclear protein involved in transcriptional regulation, DNA damage repair, and G2/M checkpoint control^{38–40}. Over 11 different splice variants of BRCA1 have open reading frames, consequently many isoforms of the BRCA1 protein have been reported in a variety of tissues⁴¹. Dynamic measurement of BRCA1 splice variants using the plasmon nanoparticle dimer approach was performed in breast cancer cell lines. Analysis of the spectral features of the dimers generated by sequence-specific binding of nanoparticle-conjugated probes at the splice junctions enabled detection of three critical BRCA1 splice variants, (9,10), (11q), and (9,10,11q), in living cells. The ratio of dimers to monomers inside the cells was used to determine variations in the presence and quantities of BRCA1 mRNA splice variants in different cell lines and at different growth stages (Figure 4). The MCF-7 and MBA-MD-231 breast cancer cell lines express the major BRCA1 splice variants, including the full length (wild-type), (9,10), (11q), and (9,10,11q) mRNAs¹. These cells are therefore appropriate for evaluating the specificity of the plasmon nanoruler approach. In addition, BRCA1 mRNA is expressed at low levels, a few tens of copies per cell³¹, in MCF-7 cells; these cells are therefore appropriate for testing the single-molecule sensitivity of the intracellular plasmon ruler concept. Based on our previous experience in quantification of these splice variants *in vitro* using SERS,^{42–44} we developed a novel *in vivo* method for the rapid screening of splice variants. Figure 4a shows detection of the BRCA1 splice variants missing the exon 9 and 10 ((9,10,11q) and (9,10)) in MDA-MB-231 cells using reconstructed images from a hyperspectral imaging scan. Images corresponding to resonant wavelengths of 612 nm and 538 nm are shown in Figures 4b and 4c, respectively (also see Movie in Supplementary Information). When the characteristic nanoparticle peaks were mapped onto the cellular images by hyperspectral imaging (with

scanning), the numbers of each mRNA splice variant could be quantified and tracked. The number of dimers increased over time; however, the increase was no longer significant after approximately 20 min (Figures 4b and 4c). As shown in Figure 1a as well as from *in vitro* verification (Figure 2) and *in vivo* calibration (Figure 3), the 612 nm scattering represents the dimer specific for the designed detection of the variants without exon 9 and 10. Results were validated with PCR experiments and verified with our previous *in vitro* quantification of these splice variants using SERS.^{42–44}

One of the most important advantages of GNP dimers is their long interaction distance. We used this property to simultaneously image and quantify (9,10,11q) and (11q) BRCA1 splice variants by measuring the spectral peaks generated by different inter-particle distances between the probes in each splice variant. Two nanoparticle-conjugated probes flanking the BRCA1 splicing junction were generated; one nanoparticle targeted the end of exon 8 and the other targeted exon 12. (Supplementary Information S12 provides a detailed account of the simulation and results). As illustrated in Figure 1b, the probe nanoparticles also attach to the wild-type BRCA1 mRNA, which makes it important to assure that our quantification was not affected by signals originating from bound monomers to wild-type mRNA. Should any signals from such dimer-like configurations on wild-type mRNA exist, it is our assumption that such non-specific conformations or targeting are negligible if not impossible. As a verification, our spectral peak histogram of the multiplex detection image (Figure 4e) displays distinct tri-modal distribution with peaks at 612 nm, 584 nm, and 538 nm representing (9,10,11q), (11q), and the nanoparticles with negligible plasmonic interaction, respectively, when the background from the cell organelles were removed (Figure 4e ii). On the other hand, the spectral peak histogram of the single target image only displays bimodal scattering peaks specific to 612 nm and 538 nm, both in our *in vitro* and *in vivo* experiments (Figure 2 and 3). Scattering peaks at other wavelengths were negligible or not observed in our experiments. A caveat is the possibility of forming 3D conformations that could potentially bring the two regions of mRNA in close proximity. However, due to the limited understanding of current state of mRNA structure and 3D conformation,⁴⁵ we are of the opinion that such errors are minimal due to the specificity of the signal and further enabled by the active unfolding of mRNA in living cells as noted from other works.⁴⁶ Detection of the two different dimers shown in Figure 4e iii)-vi) clearly highlights the merit of the plasmonic dimer probe method for the detection of multiple splice variants using a measured minimum number of probes injected into cells under physiological conditions.

Finally, we investigated the use of the plasmonic dimer method to detect the expression of three BRCA1 mRNA splice variants ((9,10), (9,10,11q) and (11q)) in different breast cancer cell lines. Figure 4f shows the differential expression levels of the alternatively spliced transcripts in MDA-MB-231 and MCF-7 cells, detected using nanoparticle-conjugated probes flanking the BRCA1 splicing junction. Since mRNA expression is dependent on the cell cycle stage, both asynchronous and cell cycle synchronized cells were examined. Analysis of the plasmon resonance spectra for each dimer enabled the detection of differential levels of BRCA1 mRNA splice variants in asynchronous cells and mimosine-treated synchronized cells (arrested in G1/S boundary) (Figure 4f, Supplementary Information S12).⁴⁷ The (9,10) and (11q) transcripts were expressed in both cell lines at

relatively high concentrations, while the (9,10,11q) variant was virtually undetectable in MDA-MB-231 cells. Importantly, there was a clear difference in the expression patterns of the splice variants between the MCF-7 and MDA-MB-231 cell lines. Triple Primer RT-PCR (TP-RT-PCR) and real-time quantitative PCR experiments performed to validate our *in vivo* quantification experiments further show that the results are highly consistent (details of PCR validation are discussed in Supplementary Information SI9).

Our findings demonstrate that the extremely low expression levels of BRCA1 splicing variants can be detected *in vivo* by plasmonic probes and hyperspectral imaging techniques, due to the single molecule sensitivity of these methods. These results are in agreement with previous data generated by PCR and SERS *in vitro*.^{42,43} In addition, control experiment with probes that do not have a target did not show any measurable dimer formation (Figure 3b inset, Supplementary Information SI8), demonstrating that the dimer formation was specific to the target mRNA splicing variants. In conclusion, the mRNA detection and quantification strategy at splice variant resolution in living single cells using plasmonic dimer probes is a highly versatile technique and has a broad appeal both in basic biology to understand gene regulation and expression and in applied research as a diagnostic tool.

Conclusion

We have shown that the plasmonic dimer strategy for hyperspectral imaging and spectral analysis can be used to detect and quantify multiple mRNA splice variants in individual living cells at single molecule precision. To this end, nanoparticle probes can be designed to hybridize to a specific mRNA and generate a characteristic signal specific to the target molecule. This information can be displayed as an image and reconstructed by a hyperspectral imaging system integrated with a dark-field microscope. Importantly, our results demonstrated that different splice variants generated unique scattering spectra, including peak shifts and intensity changes, based on dimer positioning suggesting that our platform is capable of differentiating between alternative splice variants, based on the interparticle spacing in the formed dimers. In summary, the plasmonic dimer technique provides a quantitative platform for examination of many important biological problems, including studies of biomolecular interactions, trafficking, and their kinetics in single living cells. However, in spite of the significant advantages of plasmon hyperspectral imaging in providing high SNR for single molecule detection, the long scanning time and the use of larger probes may not be applicable for observing fast molecular dynamics possible by fluorescence.^{48,49} Future work may involve the development of a fast hyperspectral imaging system for dynamic tracking and quantification of transcripts and their associated proteins in single cells at the single copy resolution.

METHODS

Detection of dimer hybridization *in vitro*

Two batches of GNP were functionalized with thiolated oligonucleotides as described in the preparation session. One sample was modified with a probe sequence (PS1) 5'-Thiol-TTTTT TTTT CGTCT GTCTA CATTG AATTG-3' (solution 1), and the other with PS2, 5'-GCTGC TTGTG AATTT TCTGA TTTTT TTTT-Thiol-3' (solution 2), of which 20

nucleotides from each probe are complementary to the 40-nt target sequence (TS1: 5'-TCAGA AAATT CACAA GCAGC CAATT CAATG TAGAC AGACG-3'). For the subsequential intracellular experiments without interference, the probe and target sequences were selected to be non-specific to the target mRNA sequences in the tested cell lines (Supplementary Information SI8). Equal proportion of the two samples (GNP-PS1 and GNP-PS2) at a concentration of 120 pM was used as the probe solution and the target oligonucleotide (TS1) was added in 40 pM concentration at a later stage. Lower concentration of the target sequence guarantees the dimer formation for molecule evaluation and prevents multimer aggregation. A dark-field microscope was used for investigation of scattering in the samples⁵⁰ after the addition of the target oligonucleotide. Cooled CCD camera was used to obtain colorimetric image of the hybridization events at 1 fps frame rate for 30 min. Images were analyzed based on the colours and intensities of particles. The colorimetric results were further validated by statistical analysis of spectral intensities of monomer and dimers measured in a small volume. Signal integration was performed only from 4×1 pixels by slit and ROI adjustment with the frame rate set at 100 ms. 100 sequential measurements every 8 minutes were obtained and analyzed to compare with the results of colorimetric images. For the multiplex experiment, we used the second target sequence (TS2: 5'-CAATT CAATG TAGAC AGACG TCAGA AAATT CACAA GCAGC-3'), which includes complementary sequences of PS1 and PS2 to form a different dimer configuration from TS1 (Supplementary Information SI7). For the control experiment, we tested nanoparticles modified with non-specific target oligonucleotide (TS3: 5'-CGTCT GTCTA CATTG AATTG GCTGC TTGTG AATTT TCTGA-3') (Supplementary Information SI6).

To show that the spectral shift and intensity change is due to the DNA hybridization, nanoparticles with PS1 sequences were fixed on the glass surface by the APTES method. The test sequence TS1 was incubated for 30 min, and nanoparticles modified with PS2 sequences were introduced and images were recorded (1 fps, 100 ms integration) to obtain snapshots of the hybridized probes at the single molecule level. After successful observation of nanoparticle dimer hybridization, the entire sample was heated to confirm that the nanoparticle dimer can be dehybridized at the calculated melting temperature of the DNA duplex.

BRCA1 splicing variants detection

GNPs were functionalized with 5'- or 3'-thiolated oligonucleotides with the following probe sequences:

PS-e8: 5'-Thiol-TTTTT TTTCA ATTCA ATGTA GACAG ACGTC TTTTG AGG -3',

PS-e11; 5'-TAC ATC CGT CTC AGA AAA TTC ACA AGC AGC TTT TTT TT - Thiol-3',

PS-e12: 5'-TTT CAC TCT CAC ACC CAG ATG CTG CTT CAC TTT TTT TT - Thiol-3'.

PS-e8, PS-e11 and PS-e12 are targeting 3'-end of the exon 8, 5'-end of the exon 11, and 5'-end of the exon 12, respectively. Mixture of Monomer particles modified with probe

sequences, GNP-PS-e8 and GNP-PS-e11 in the concentration of 120 pM, were injected into the cytosol to quantify the BRCA1 alternative splicing variants missing the exon 9 and 10. Similarly, mixture of GNP-PS-e8 and GNP-PS-e12 was utilized for multiplex quantification of the BRCA1 alternative splicing variants (11q) and (9,10,11q). After the quantification of (9,10,11q), the quantification of (9,10) was calculated by subtracting the number of (9,10,11q) from that of variants missing the exon 9 and 10. To separate different mRNA expression depending on the cell growth stages, cells were synchronized at G1/S boundary by treating with 200 uM mimosine in the culture medium for 12 hours.⁴⁷ Cell Synchronization experiments were conducted by mimosine treatment and validated by flow cytometry (FC500MPL, Beckman Coulter Inc., Brea, CA) (Supplementary Information SI12). Quantification of the variant (9,10) and multiplex experiments to detect variants (11q) and (9,10, 11q) were repeated after cell growth synchronization. To validate our result, simulation of mRNA secondary structure was performed (Centroidfold, <http://www.ncrna.org/centroidfold/>) as appropriate to highlight the differences in secondary structures and details are provided in Supplementary Information SI2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic design of sequence-selective single mRNA detection. a) Spectral characteristics of different nanoparticle structures formed by DNA hybridization for sequence-specific detection. Nanoparticle probes targeting different exons in the mRNA are highlighted by different colors. Monomers (light blue line) and dimers (purple line) can be differentiated from one another due to their unique spectral characteristics. Inset: Real color images (left panel) and finite-difference time-domain (FDTD, right panel) simulation results of the nanoparticle monomer (i) and dimer (ii) showing the field intensities with (dimer) and without (monomer) plasmonic coupling between the particles. Scale bar: 1 μm in real color images and 20 nm in FDTD simulation results. b) Nanoparticle probes are injected into individual living cells to form dimers in a sequence-specific manner upon targeting a single mRNA. Hyperspectral imaging presents the full spectrum generated by each point in the whole image, so that splicing information can be analyzed based on the changes in the spectral profile. Examples of representative BRCA1 splicing structures (certain sequences are missing from wild-type) and their spectral features when they are detected by nanoparticle dimers are shown.

Figure 2.

Hybridization dynamics of nanoparticle monomers and dimers measured by imaging and spectral analysis. Target molecules are quantified by the number of dimers formed *in vitro*. a) Dark-field images of nanoparticles after addition of target molecules, show dimerization of nanoparticles over time. Bright yellow spots represent nanoparticle dimers; dim green spots represent nanoparticle monomers. b, c) Spectral quantification of monomers and dimers, respectively, measured in relatively noise-free region (red square in (a)). Spectra measured on 4 pixels of a CCD camera reveal intensity variation at (b) 538 nm and (c) 612

nm, for monomer and dimer signals, respectively. The variation follows Poisson distribution, providing the average number of particles in a confined volume. d) Quantification of images and spectral data show an inverse trend in the levels of monomers (blue dashed line) and dimers (red solid line). e) Single molecule detection captured by CCD images (1 fps, 100 ms integration). Two monomers (red arrows) in close proximity ((i)-(iii)) and formation of a dimer (iv). f) When the temperature is increased, the nanoparticle dimer is disassembled at the melting temperature of the hybridized DNA, 64.7°C.

Figure 3.

a) Approximately 12.5, 25, 37.5, or 50 dimers are injected into four different cells by microinjection, respectively (i-iv). Dark field image (left panel) and dimer localization map from hyperspectral image (right panel) of each cell are presented. Scale bar: 10 μm . b) Calibration of the whole-cell spectral quantification technique. Inset): Dimer peak intensities of calibration (dimers) and control experiments (monomers only) are plotted after the baseline was subtracted. Error bars indicate the standard deviation of five independent measurements.

Figure 4.

Detection and quantification of mRNA by hyperspectral measurement of plasmonic dimer probes. Red dash line represents the cell borders. Scale bar: 10 μm . a) Hyperspectral image of synchronized MDA-MB-231 cells containing injected nanoparticle probes. b, c) BRCA1 alternative splicing variants missing exon 9 and 10 are targeted by plasmonic nanoparticle probes, and hyperspectral images of (b) dimers (red dots) and (c) monomers (green dots) at several time-points are shown. Scale bar: 10 μm . d) A distinct increase in the number of dimers was observed over time. Number of dimers represent the quantification of the target variants missing exon 9 and 10. Error bars indicate the standard deviation of measurements in three different cell lines. Inset) Wavelength window (blue column) for the dimer detection corresponding to the dimer spectrum (solid purple line) and the description of length change by splicing event. Wavelength window (red column) for the monomer detection (light blue

dashed line) was also presented. e) i) Multiplex detection of (9,10,11q) and (11q) using a single set of probes in MCF-7 cells. ii) The tri-modal distribution of the detected plasmon peaks, showing the existence of only two different dimer structures (signals at 584 nm and 612 nm corresponding to (11q) and (9,10,11q), respectively), exhibits minimal interference at other wavelengths possibly indicating that non-targeted mRNA isoforms have negligible plasmonic interaction. Corresponding dimer localization maps, (iii) and (v), as well as their wavelength widows and splicing variant conformations, (iv) and (vi), are presented. Scale bar: 10 μm . f) Single cell spectral quantification of three splicing variants (9,10), 11q and (9,10, 11q) for the two cell lines, MCF-7 and MDA-MB-231, at asynchronous and G1/S synchronized growth stages.